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1) J Chromatogr 1990 Feb 23;525(2):297-306
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Toki N, Takasugi S, Sumi H.

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Miwa N, Takayanagi H, Suzuki A.

Thank you,
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Purification of High Mol cular Weight Urokinas from Human Urine and Comparative Study of Two Activ Forms of Urokinase

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Key words

Purification – High and low molecular weight urokinase – Fibrinolysis – Fibrin-tube method – Fibrin-plate method – Two-stage lysis time method – Esterolytic activity

Summary

An improved method for the purification of high molecular weight urokinase to homogeneity from human urine was established. A yield of 32% with a 3,100-fold purification was obtained by Hyflo Super-Cel treatment, heat treatment at 60°C for 10 hr, serial column chromatography on DEAE-Sepharose CL-6B and O-[3-(p-sulfophenylamino)-2-hydroxypropyl]-cellulose (SFOP-cellulose), and gel filtration on Ultrogel AcA 54. The low molecular weight form of urokinase was also purified to homogeneity by chromatography on hydroxyl apatite and gel filtration on Sephadex G-75 after the SFOP-cellulose column step. The high molecular weight urokinase had only one isoelectric form with a pI of 9.7, whereas the low molecular weight form had six isoelectric subforms with pI values between 9.4 and 6.4. The absorption coefficients at 280 nm of both urokinase forms were 13.61 and 13.50, respectively. Fibrinolytic and esterolytic activities of the two urokinase forms were compared in various assay methods.

Introduction

Urokinase (EC 3.4.21.31) is a plasminogen activator synthesized in mammalian kidney and released into urine (1–6). Urokinase is a serine protease specific for the conversion of plasminogen to plasmin, the protease responsible for the degradation of fibrin. Due to its thrombolytic properties, urokinase is used therapeutically to promote dissolution of thrombi in vivo.

Two active forms of urokinase with molecular weights of 47,000–55,000 and 31,000–34,000 have been isolated and characterized (7–14). The low molecular weight urokinase is a degradation product of the high molecular weight forms (11). The latter is superior to the low molecular weight form in terms of fibrinolytic activity against thrombi, stability in vivo, and affinity to fibrin (15, 16). The yields of high molecular weight urokinase are only 10% or less (10, 11, 14).

This report describes an efficient large scale purification method of high molecular weight urokinase and compares some properties of the high and low molecular weight forms.

Abbreviations:
MCA: 7-amino-4-methylcoumarinamide; pNA: p-nitroanilide; SFOP: 3-(p-sulfophenylamino)-2-hydroxypropyl; SDS: sodium dodecyl sulfate.

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Materials and Methods

Determination of urokinase activity. During the purification, the plasminogen activator activity of urokinase was determined in terms of fibrinolytic activity by means of the fibrin-tube method of Ploug and Kjeldgaard (17) using the International Standard Preparation supplied by the National Institute of Hygienic Sciences, Tokyo (Japan) (MM 004: 1,000 IU/vial) for calibration. The fibrin-plate method (17) and the two-stage lysis time method (18) were adopted for the determination of the specific activity of the purified urokinase. Kinetic studies using synthetic substrates, glutaryl-glycyl-L-arginine-MCA (Protein Research Foundation, Osaka, Japan) and pyroglutamyl-glycyl-L-arginine-pNA (Kabi S-2444) were carried out by the methods of Morita et al. (19) and Claeson et al. (20), respectively.

Protein determination. The method of Lowry et al. (21) with bovine serum albumin as a standard was used.

Polyacrylamide gel disc electrophoresis. SDS-polyacrylamide gel disc electrophoresis was carried out in 5×90 mm columns at a polyacrylamide gel concentration of 7.5% at pH 7.1, using the procedure of Weber and Osborn (22). Protein was stained with 0.05% Coomassie brilliant blue in 12.5% trichloroacetic acid.

Isoelectric focusing. The procedure of Vesterberg and Svensson (23) was used.

Molecular weight determination. The molecular weight of samples was determined by SDS-polyacrylamide gel disc electrophoresis and by gel filtration on a 2.5×90 cm column (Pharmacia Fine Chemicals) of Sephadex G-75 equilibrated with 0.05 M Na_2-K-phosphate buffer, pH 7.0, containing 0.5 M NaCl. The following proteins were used as references: bovine serum albumin (Mr 67,000), ovalbumin (Mr 45,000), bovine pancreatic chymotrypsinogen A (Mr 25,000), and horse heart cytochrome c (Mr 12,500).

Preparation of SFOP-cellulose. SFOP-cellulose was prepared by the method of Porath and Fornstedt (24). The yield and capacity of SFOP-cellulose was 88% and 420 µmoles per g of dry weight, respectively.

Purification of high molecular weight urokinase. All steps except mentioned were performed at 15°C.

Step 1: adsorption to Hyflo Super-Cel. Treatment with Hyflo Super-Cel was performed by the method of Bergström (25). Fresh urine (1,500 l) from normal healthy human males containing 1 ml of toluene per liter was adjusted to pH 5.0 with 6 N HCl. For each liter of urine, 10 g of Hyflo Super-Cel were added and the suspension was stirred for 30 min and allowed to stand overnight in a cold room (4°C) to settle the Hyflo Super-Cel. The adsorbent was collected on Buchner funnel and washed with distilled water until the wash solution was colorless. The urokinase was eluted with 1 N NH_4OH and the pH of the elute was adjusted to 8.0 by dropwise addition of 3 N H_2SO_4. The mixture was allowed to stand for 2 hr and the mucous precipitate formed was removed on a Buchner funnel.

Step 2: salting out with ammonium sulfate. To each liter of the supernatant solution (124 l) of *step 1*, 450 g of $(NH_4)_2SO_4$ were added with stirring, and the mixture was allowed to stand overnight in a cold room (4°C). The precipitate formed was collected by centrifugation and dissolved in a small amount of 0.1 M Na_2-K-phosphate buffer, pH 7.2. The solution was desalted with polysulfone hollow fibers (Asahi Kasei Kogyo Co., Labomodule SIP-1013, 0.8 mm inner diameter ×25 cm length ×400 fibers, filtration area 0.25 m^2, flow rate 270 ml/min, filtration rate 90 ml/min).

Step 3: column chromatography on Sephacryl S-200. The crude urokinase solution (3,400 ml) of *step 2* was passed through a column

Table 1 Purification of urokinase

Step	Volume ml	Protein mg	Urokinase IU	IU/mg	%	Mr of Urokinase
			$\times 10^4$			
1. Urine	1,500,000	180,000	795.00	44	100.0	
2. Hyflo Super-Cel	124,000	43,722	657.00	150	82.6	
3. $(NH_4)_2SO_4$ 0.85	3,400	26,588	632.40	238	79.5	
4. Sephacryl S-200	5,420	408.7	631.97	15,464	79.5	
5. Heat treatment	40	404.0	512.4	12,683	64.5	54,000
6. DEAE-Sepharose-SFOP-cellulose	110	46.42	316.03	68,080	39.8	
7. Ultrogel AcA 54	30	19.05	259.11	136,015	32.6	
8. Desalination	22	18.92	257.18	135,930	32.3	
6. SFOP-cellulose	300	76.68	639.00	83,330	100.0	
7. Hydroxyl apatite	120	34.15	459.06	134,420	71.8	
8. Sephadex G-75	30	9.57	211.82	220,980	33.1	33,000
9. Desalination	22	9.00	198.45	220,500	31.1	

(12×66.3 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Sweden) equilibrated with 0.01 M Na_2-K-phosphate buffer, pH 6.0. The column was washed with the same buffer used for equilibration of the resin followed by 0.01 M Na_2-K-phosphate buffer, pH 8.0, until the absorbancy of the effluent at 280 nm became less than 0.01. Urokinase was then eluted from the column with 0.8 M NaCl in 0.01 M Na_2-K-phosphate buffer, pH 8.0. This active fraction (5,420 ml) was concentrated to 40 ml with polysulfone hollow fibers.

Step 4: heat treatment. To the concentrated urokinase solution (40 ml), 40 ml of 2% hydroxypropylcellulose (Nippon Soda Co., HPC-SL, Mr less than 30,000) dissolved in 0.3 M ammonium phosphate buffer, pH 7.4, were added, and the mixture was incubated at 60°C for 10 hr.

Step 5: serial column chromatography on DEAE-Sepharose CL-6B and SFOP-cellulose. The mixture was diluted 20-fold with 0.01 M Na_2-K-phosphate buffer, pH 8.0, lowering the conductivity of the solution to less than 10 mmho per cm, and applied to serial columns of DEAE-Sepharose CL-6B (2.6×7.6 cm) and SFOP-cellulose (2.0×12 cm). The flow rate during column loading and washing with 0.03 M NaCl in 0.01 M Na_2-K-phosphate buffer, pH 8.0, was about 160 ml/hr. DEAE-Sepharose CL-6B does not adsorb urokinase but adsorbs pyrogen effectively. SFOP-cellulose adsorbs all types of urokinase, but the strength of adsorption varies depending on the molecular type of urokinase. Washing of the SFOP-cellulose column with 0.09 M NaCl in 0.01 M Na_2-K-phosphate buffer, pH 8.0, eluted the low molecular weight urokinase and traces of pyrogen, but did not elute the high molecular weight urokinase. The high molecular weight urokinase was eluted from SFOP-cellulose by 0.5 M NaCl in 0.01 M Na_2-K-phosphate buffer, pH 8.0.

Step 6: gel filtration on Ultrogel AcA 54. The active fractions (110 ml) of the SFOP-cellulose column chromatography were concentrated to 5 ml with polysulfone hollow fibers (0.8 mm×100 cm) and applied to an Ultrogel AcA 54 column (1.7×52.9 cm) equilibrated with 1 M NaCl in 0.01 M Na_2-K-phosphate buffer, pH 7.0. The flow rate was about 120 ml/

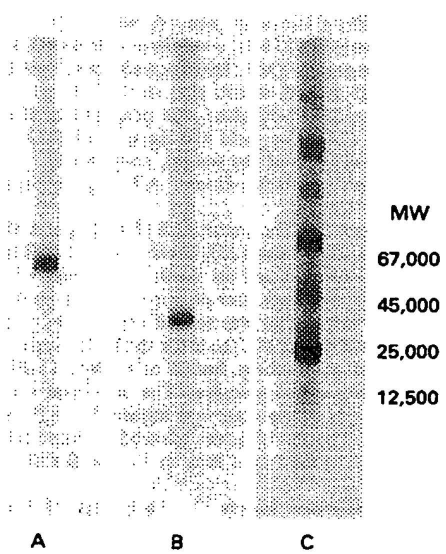

Fig. 1 SDS-polyacrylamide gel disc electrophoresis of high and low molecular weight urokinase. Electrophoresis was carried out at 8 mA for 4 hr, using 20 µg fo each sample. (A) high molecular weight urokinase, (B) low molecular weight urokinase, (C) bovine serum albumin, ovalbumin, bovine pancreatic chymotrypsinogen A, and horse heart cytochrome c.

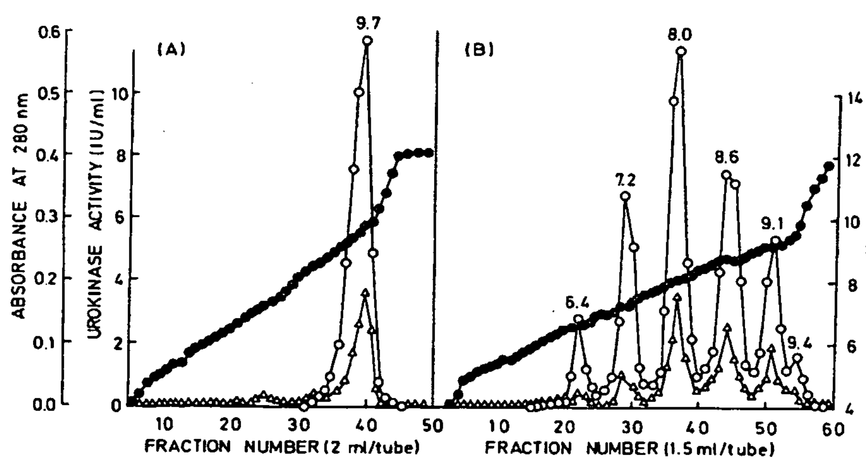

Fig. 2 Isoelectric focusing of high and low molecular weight urokinase. (A) 0.850 mg high molecular weight urokinase (114,600 IU, 134,800 IU/mg) or (B) 0.954 mg of low molecular weight urokinase (210,400 IU, 220,500 IU/mg) was applied to an electrofocusing column (110 ml), using Ampholine pH 3.5–10 (LKB Produkter AB) as carrier ampholyte. Electrofocusing was performed at 4°C for 72 hr with a constant potential of 600 V (initial current: 4.1 mA, final current: 1.6 mA). Symbols: ○, urokinase activity by fibrin-tube method; △, absorbance at 280 nm; ●, pH.

hr (SV = 1.0). The active fractions (30 ml) obtained were desalted and concentrated to 22 ml with polysulfone hollow fibers. Thus, pure high molecular weight urokinase was obtained.

Purification of Low Molecular Weight Urokinase

Step 1: chromatography on hydroxyl apatite. The starting material was the low molecular weight urokinase fraction eluted from SFOP-cellulose during the purification of high molecular weight urokinase. This fraction was desalted and concentrated to 50 ml with polysulfone hollow fibers and applied to a column of hydroxyl apatite (1.28×7.7 cm). The column was washed with 0.01 M Na_2-K-phosphate buffer, pH 8.0. Urokinase was eluted with 0.15 M NaCl in 0.01 M Na_2-K-phosphate buffer, pH 8.0. The active fractions (110 ml) with specific activities of more than 130,000 IU/mg of protein were collected and concentrated to 12 ml with polysulfone hollow fibers.

Step 2: gel filtration on Sephadex G-75. The concentrated fractions (12 ml) of *step 1* were applied to a column of Sephadex G-75 (2.6×90 cm) equilibrated with 1 M NaCl in 0.01 M Na_2-K-phosphate buffer, pH 7.0. The flow rate of the elution buffer, the same as the equilibrating buffer, was 25 ml/hr (SV = 0.05). The active fractions (30 ml) were desalted and concentrated to 22 ml with polysulfone hollow fibers. Thus, pure low molecular weight urokinase was obtained.

Results

Purification of urokinase. The yields and specific activities of the various fractions obtained during the course of the purification of the enzyme are listed in Table 1. From the initial 1,500 l of urine containing 795×10^4 IU of urokinase, 257×10^4 IU of high molecular weight urokinase and 13.2×10^4 IU of low molecular weight urokinase were obtained. The procedure results in a 3,100-fold purification of high molecular weight urokinase with an overall recovery of 32.3%. The absorption coefficients, $A_{1\,cm}^{1\%}$ at 280 nm at pH 7.0 in 0.01 M Na_2-K-phosphate buffer containing 1 M NaCl, of the high and low molecular weight urokinase were 13.61 and 13.50, respectively.

Homogeneity. The homogeneity of the purified enzymes was examined by SDS-polyacrylamide gel disc electrophoresis at pH 7.1 (Fig. 1). Under non-reducing conditions only one single protein band appeared for both high and low molecular weight urokinase, corresponding to molecular weights of 54,000 and 33,000, respectively.

Molecular weight determination by gel filtration. The molecular weight of the two forms of native urokinase, estimated by gel filtration on Sephadex G-75 were 54,000 and 33,000, respectively.

Isoelectric focusing. Fig. 2 shows the results of isoelectric focusing of the two purified forms of urokinase. High molecular weight urokinase showed a single peak at pH 9.7, while the low molecular weight form had six isoelectric subforms with a pI between 6.4 and 9.4.

Stability and molecular conversion of the purified urokinase. Fibrinolytic activities of the two forms of urokinase were quite stable at 4°C for several months, and at −14°C ~ −80°C for at least one year. On prolonged storage at 4°C high molecular weight urokinase is converted autocatalytically to a low molecular weight form. The conversion occurs more easily at lower ionic strength and at higher pH or temperature. On incubation in 0.01 M Na_2-K-phosphate buffer, pH 8.0, at 20°C for 1, 3, and 10 days, 8, 20, and 55% conversion was observed, respectively, as monitored by gel filtration on Sephadex G-75. The molecular weight conversion did not occur below −14°C, or in 0.01 M phosphate buffer, pH 7.0, containing 1 M NaCl at 4°C for 10 days. Both forms of urokinase lost their fibrinolytic activity below pH 6.0 or above 55°C. Albumin, gelatin, or hydroxypropyl cellulose protect urokinase against heat inactivation at 60°C for 10 hr.

Activities of the two forms of urokinase in various assay systems. Fibrinolytic activities of the two forms of urokinase were estimated and expressed as international unit; IU, and summarized in Table 2.

Kinetic studies of two forms of urokinase. Esterolytic activities of the two forms of urokinase were estimated with glutaryl-glycyl-L-arginine-MCA and pyroglutamyl-glycyl-L-arginine-pNA. The results are summarized in Table 3. Turnover numbers and K_m values of the two forms of urokinase were the same.

Table 2 Catalytic activities in various assay methods

Method	Catalytic activity		Mr 54,000	Mr 33,000
Fibrin-tube (T)	Specific activity T/TS	(IU/mg) (%)	134,809 122.5	220,503 101.6
Two-stage lysis time (TS)	Specific activity TS/T	(IU/mg) (%)	107,420 79.7	220,117 98.5
Fibrin-plate (P)	Specific activity P/T	(IU/mg) (%)	113,719 84.4	223,320 101.3
MCA	Specific activity MCA/T	(IU/mg) (%)	105,155 78.0	172,138 78.1
pNA	Specific activity pNA/T	(IU/mg) (%)	111,247 82.5	182,075 82.6

Table 3 Kinetic parameters of urokinase for synthetic substrates

Substrate	Parameter	Mr of urokinase 54,000	33,000
Glutaryl-gly-L-arg-MCA	V_{max} (μmoles/min/mg protein)	19.31	32.94
	Turnover number (moles/min/moles enzyme)	1,042	1,087
	K_m (μM)	265	264
Pyroglutamyl-gly-L-arg-pNA	V_{max} (μmoles/min/mg protein)	32.42	52.42
	Turnover number (moles/min/moles enzyme)	1,750	1,730
	K_m (μM)	75	76

Discussion

The purpose of this study was to establish a large scale preparation of electrophoretically homogeneous high molecular weight urokinase. This was achieved by the method described; a 3,100-fold purification and a yield of 32% were realized.

The gels and adsorbents used for the purification of high molecular weight urokinase, Sephacryl S-200, DEAE-Sepharose CL-6B, SFOP-cellulose, and Ultrogel AcA 54 could be used repeatedly, at least 50 times. Sterilization and removal of pyrogen from these media was easily performed. Sephacryl S-200, DEAE-Sepharose CL-6B and SFOP-cellulose were stable during autoclaving at 120°C for 60 min. Ultrogel AcA 54 and also the other adsorbents could be sterilized with 0.5% chlorhexidine and pyrogen was removed from the resins with 0.5% sodium deoxycholate followed by washing with 20 volumes of 0.2 N sodium hydroxide and 0.2 N hydrochloric acid.

Holmberg et al. (10) and Soberano et al. (11) purified urokinase by affinity chromatography on agmatine-Sepharose or benzamidine-Sepharose. These methods afforded an efficient purification of fibrinolytic activity but the separation of the two forms of urokinase was insufficient and proteolytic degradation occurred frequently during the course of purification. According to our experience SFOP-cellulose is an efficient adsorbent for high molecular weight urokinase, while low molecular weight urokinase and pyrogen were eluted at low ionic strength.

Any viruses which might contaminate the urine are expected to be inactivated by heat treatment at 60°C for 10 hr (26). In the presence of hydroxypropyl cellulose, fibrinolytic activity remained stable and the degradation to low molecular weight urokinase did not occur during heat treatment. Hydroxypropyl cellulose was easily removed from the urokinase solution, since this compound is uncharged and is, therefore, not adsorbed to DEAE-Sepharose and SFOP-cellulose.

According to unreduced SDS-polyacrylamide gel electrophoresis and gel filtration the apparent molecular weights of the two purified forms of urokinase are 54,000 and 33,000. These values are similar to those of Nobuhara et al. (12) (51,600 and 34,000), Miwa et al. (13) (55,000 and 36,000), Holmberg et al. (10) (54,000), and Soberano et al. (11) (33,000). The yield (32.3%) of high molecular weight urokinase from human urine by the present procedure is higher than those ever described (Nobuhara's method, 8.0% and Miwa's method, <10.2%). Thus, the purification procedure described here provides the basis for a large scale preparation of electrophoretically homogeneous high molecular weight urokinase from human urine.

As a by-product, we also purified low molecular weight urokinase to electrophoretic homogeneity, with molecular parameters mentioned above.

Some properties of both forms of urokinase were compared. High molecular weight urokinase gave a single peak upon isoelectric focusing, whereas low molecular weight urokinase gave six peaks. A single form of the high molecular weight urokinase with a pI value of 9.7 was reported by Nobuhara et al. (12), whereas multiple forms of high molecular weight urokinase was reported by Soberano et al. (11) and Miwa et al. (13). Multiple isoelectric forms of low molecular weight urokinase was found by Soberano et al. (11), Ogawa et al. (9), Miwa et al. (13), and Nobuhara et al. (12).

Fibrinolytic activities of the two types of urokinase differ between various reports, making a comparison of values difficult.

The specific activities of electrophoretically homogeneous urokinase can be expressed precisely in terms of the parameters obtained with synthetic substrates. The V_{max} values of our preparations of high and low molecular weight urokinase were 19.3 and 32.9 with glutaryl-glycyl-L-arginine-MCA, and 32.4 and 52.4 with pyroglutamyl-glycyl-L-arginine-pNA, respectively. Turnover numbers and K_m values, respectively, were practically the same for the two urokinase forms. Both of them had a higher affinity and a higher V_{max} for the pNA than for the MCA substrate.

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